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14. ABSTRACT With the development of new methodologies has come a greater appreciation of how genes are able to interact with distal regulatory elements. Gene transcription may be regulated by remote enhancer regions through chromosome looping. The role that higher order chromatin organization plays in cancer progression and metastasis is not yet fully understood. Our major goal has been to characterize physical interactions among selected breast cancer gene loci in normal and malignant mammary cell lines. During the past year, we used IGFBP3, a gene that has been involved in breast cancer pathogenesis, as bait in a 4C-seq experiment comparing normal breast cells (HMEC) and two different breast cancer cell lines (MCF7, an ER positive cell line and MDA-MB-231, a triple negative cell line). We found that in HMEC the genes BCAS1-4 (breast carcinoma amplified sequence 1-4) were found in the top 10 most significantly enriched regions for interactions with IGFBP3. We also found EGFR (epidermal growth factor receptor), a gene that has also been implicated in cancer, to interact significantly with IGFBP3 in all three samples. These data suggest an important role for chromosomal interactions in the pathogenesis of breast cancer.					
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Introduction

Nuclear architecture is the new dimension of regulatory control, functioning in conjunction with genome organization and epigenetic marks. A full understanding of a cell's genetic repertoire cannot be discerned from linear sequence analysis alone. Instead, we must have a full understanding of the three dimensional nature of the human genome. Dynamic interactions occur among DNA elements, which can regulate gene expression over large genomic distances on a single chromosome, through DNA looping, or even between chromosomes. We propose that incorporating new knowledge regarding a breast cancer gene's spatial interactions (i.e., the nuclear neighborhood within which the genes reside) will yield novel and more accurate predictions of breast cancer susceptibility and suggest innovative therapeutic options.

Body

Task 1: Characterize physical interactions between selected breast cancer loci in normal and malignant mammary cell lines. (Months 1 - 24)

Insulin-like growth factor binding protein 3 (IGFBP3) has been implicated in breast cancer pathogenesis (1-5). IGFBP3 modulates cell growth and survival through binding to insulin-like growth factors I and II, and regulating their bioavailability (6). IGFBP3 has also been proposed to function independently of IGF and act as a growth modulator (7-9). While correlations between serum levels of IGFBP3 and breast cancer have yielded contradictory results (3-5, 10), increased levels of IGFBP3 in breast cancer tissue is correlated with a worse prognosis and poor clinical features (1,2).

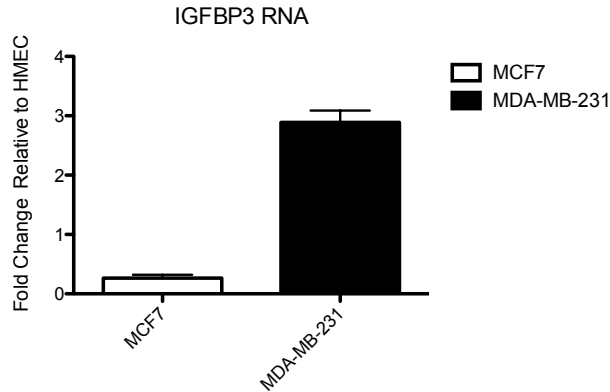
Dysregulation of IGFBP3 expression and hypermethylation of its promoter have been observed in many cancers (11). High levels of IGFBP3 expression was observed to increase survival of breast cancer cells exposed to environmental stress (12). We hypothesized that cancer-related changes in IGFBP3 regulation might coincide with altered spatial positioning and long-range DNA interactions contributing to breast cancer pathogenesis. We therefore used the IGFBP3 enhancer as bait in circular chromosome conformation capture with high throughput sequencing (4C-seq) in normal mammary epithelial cells (HMEC) and two breast cancer cell lines, MCF7 and MDA-MB-231, with opposite IGFBP3 expression profiles.

Expression of IGFBP3 is downregulated in MCF7, but upregulated in MDA-MB-231 relative to HMEC.

To better understand the role of IGFBP3 in breast cancer we analyzed its expression in primary breast cells, the estrogen receptor alpha (ER α) positive breast cancer cell line MCF7, and the triple-negative breast cancer cell line MDA-MB-231. IGFBP3 expression was increased nearly 3-fold in MDA-MB-231, and reduced 3.8-fold in MCF7, relative to HMEC (figure 1A). To evaluate whether DNA methylation correlated with the changes in expression, we examined the methylation status of the IGFBP3 promoter by bisulfite pyrosequencing. The IGFBP3 promoter was hypermethylated (91% CpG methylation) in MCF7

compared with 11% and 10% CpG methylation in HMEC and MDA-MB-231, respectively (figure 1B).

A



B

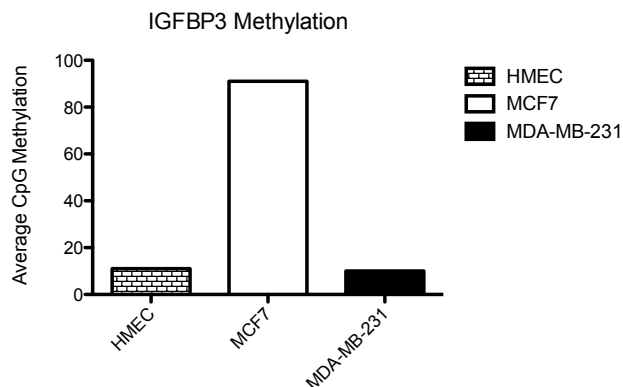


Figure 1. Expression and methylation status of IGFBP3

A) qRT-PCR: RNA levels of *IGFBP3* were measured in MCF-7, MDA-MB-231 and HMEC cells. Expression in cancer lines was plotted as fold change relative to HMEC. Data represent the SEM of three independent biological replicates. B) Percent methylation of CpG nucleotides in the *IGFBP3* promoter in HMEC, MCF-7 and MDA-MB-231. Bars represent the average percent methylation of 2-6 positions in the *IGFBP3* promoter.

EGFR interacts significantly with IGFBP3

To identify whether changes in IGFBP3 expression and methylation were accompanied by global alteration of its long-range chromatin interactions, we performed multiplex 4C-seq in HMEC, MCF7 and MDA-MB-231. We chose a

region upstream of *IGFBP3*, classified as a strong enhancer in HMEC by chromatin profiling of several distinctive features including enrichment of the enhancer mark H3K4me1 (13), as our bait. We obtained a combined total of approximately 12 million mapped reads for the three samples with the majority mapping in cis. The 4C-seq reads were binned into windows based on the number of mappable HindIII restriction sites ranging from 25 to 400. Regions with a FDR below 0.01 were considered significantly interacting. The significant long-range cis interactions for window size 100 in HMEC, MCF7 and MDA-MB-231 are diagrammed (Figure 2A). For every window size analyzed, MCF7 contained the largest number of significant interactions, followed by MDA-MB-231 and HMEC. Within a window size of 100, there were a total of 16 significant cis interactions in HMEC, 51 in MCF7 and 29 in MDA-MB-231. Of these interactions 8 were common to all samples.

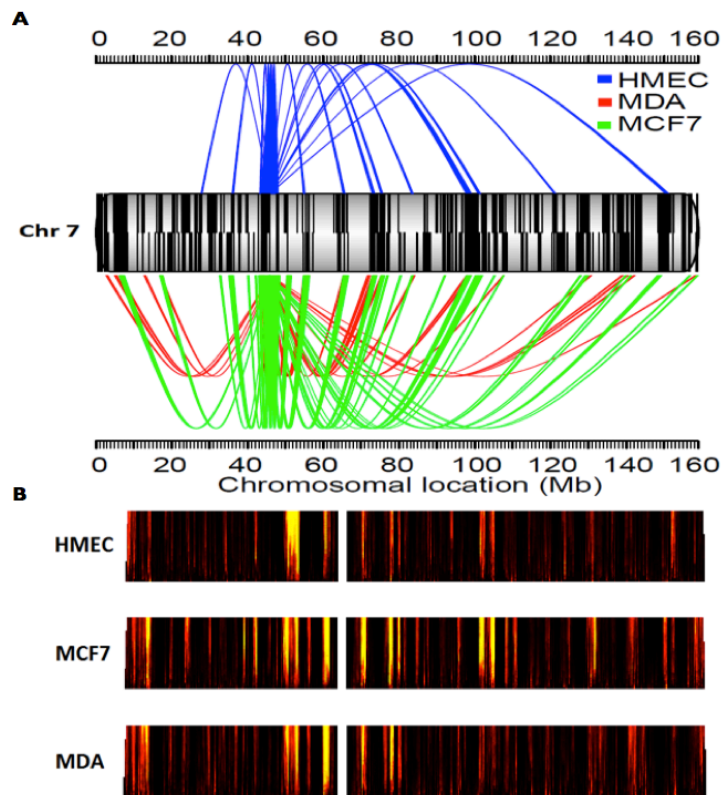


Figure 2. Intrachromosomal Interactions of *IGFBP3* A) Spider plot showing the significant long-range interactions of the *IGFBP3* enhancer across chromosome 7 in HMEC (blue), MDA-MB-231 (red), and MCF7 (green). Mb position is plotted. Tick marks on chromosome 7 represent gene locations with positive strand genes on top and negative strand genes on the bottom.

Among the significant intrachromosomal interactions common to all samples, and across all window sizes, was an interaction with epidermal growth factor receptor (EGFR), another breast cancer related gene. EGFR is located approximately 9 Mb from *IGFBP3* on chromosome 7. To examine this long-range interaction in

more detail, we labeled gene pairs *EGFR* and *IGFBP3* by 3D-FISH in HMEC and breast cancer cell lines MCF7 and MDA-MB-231 (figure 3A). To quantitate differences in interaction frequencies at the cellular level, we measured the center-to-center distances between the closest pairs of labeled foci. In 88% of HMEC nuclei counted *EGFR* and *IGFBP3* were within 1 micron (Figure 3B). This was reduced to 56% of MCF7 nuclei, and increased to 96% of MDA-MB-231 nuclei. To assess whether differences in spatial positioning were accompanied by changes in expression, we measured RNA levels of *EGFR* in HMEC, MCF7 and MDA-MB-231 by qRT-PCR (Figure 3C). Relative to HMEC, *EGFR* expression was unchanged in MDA-MB-231, yet was reduced 35-fold to nearly undetectable levels in MCF7 cells. In contrast to *IGFBP3*, the *EGFR* promoter had no change in CpG methylation (data not shown).

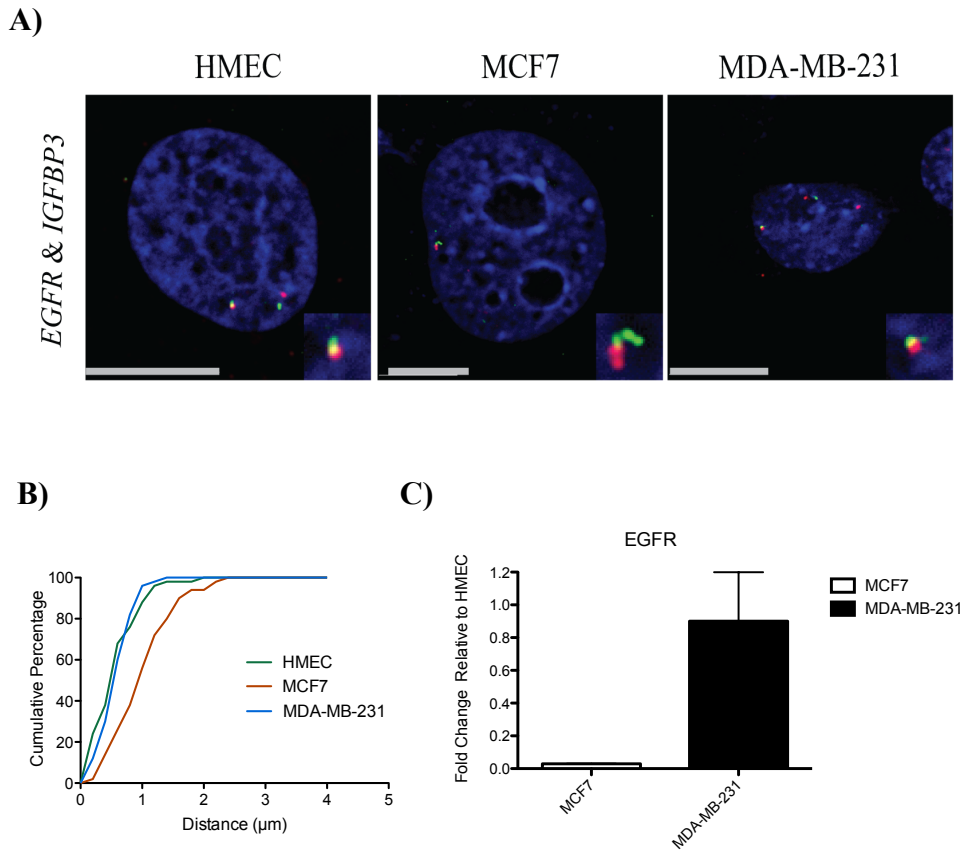
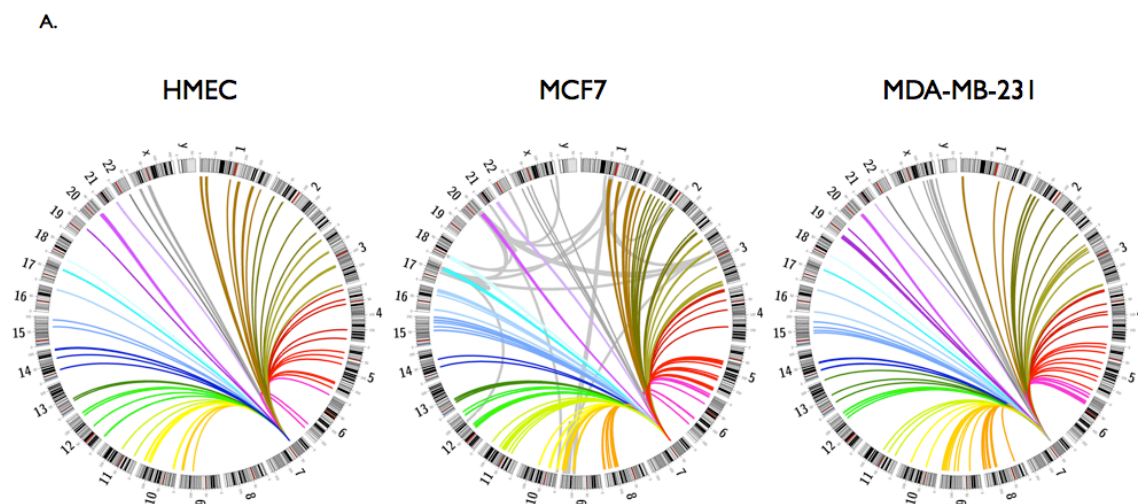


Figure 3. Interaction frequency of *IGFBP3* with the breast cancer related gene *EGFR* by 3D-FISH.

A) 3D-FISH labeling of breast cancer related loci in HMEC, MCF7, MDA-MB-231. BAC probe combinations: *IGFBP3* (green) and *EGFR* (red) $n=50$, DAPI DNA stain (blue), boxes in lower right corner contain a magnified view of each interaction. Scale bar = 10 μm . B) Cumulative percentage of distances between *IGFBP3* and *EGFR* loci. Distances were measured between the closest two foci in each nucleus. C) qRT-PCR: RNA levels of *EGFR* measured in MCF-7, MDA-MB-231 and HMEC cells. Expression in cancer lines plotted as fold change relative to HMEC. Data represent the SEM of three independent biological replicates.

Recurrent breakpoints that map within HMEC 4C significant hits are also present within MCF-7 4C significant hits

We constructed a circus plot to highlight the significant interchromosomal interactions involving the IGFBP3 enhancer in HMEC, MCF7 and MDA-MB-231 that fell within a window size of 200 (Figure 4A). There were a total of 87 significant interactions in HMEC, 194 in MCF7 and 115 in MDA-MB-231. Of these interactions only 11 were common to all samples (figure 4B, Table S3). Because a large proportion of the significant 4C windows fell within chromosomes prone to rearrangements, fusions and amplifications, we compared the locations of a list of 157 breakpoints mapped in MCF7 cells (36) to our significant interchromosomal 4C windows. The breakpoints could be categorized as 2 distinct types. The first category contained the majority of breakpoints, which were dispersed throughout the genome in regions of low copy repeats. The second category included MCF7 breakpoints falling within four highly amplified regions located on chromosomes 1, 3, 17 and 20. We found that breakpoints falling within our 4C windows were almost exclusively in the latter category. We considered a subset of 74 breakpoints, described as interchromosomal rearrangements, and determined how many of these fell within significant 4C windows in MCF-7. As a comparison we also mapped these breakpoints to our significant 4C windows in HMEC. A total of 29 breakpoint ends mapped within significant windows in HMEC, as compared to 61 in the MCF-7 line. Interestingly, all but 1 of the breakpoints within HMEC 4C windows was also present within MCF-7 4C hits. Also, when we compared the number of breakpoints of which both ends of the breakpoint mapped to a 4C hit, the percentage was nearly twice as many in the breast cancer cell line MCF-7 as in HMEC.



B)

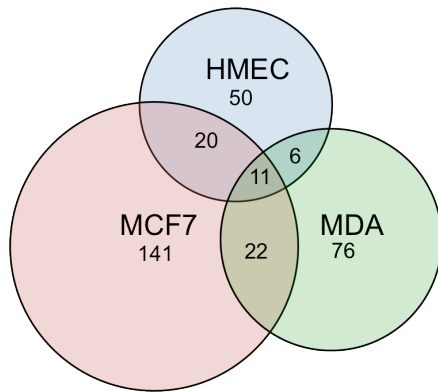


Figure 4. Distribution of interchromosomal Interactions

A) Circos plot showing the distribution of significant interchromosomal interactions involving *IGFBP3* in HMEC, MCF7 and MDA-MB231. Grey lines in MCF7 plot represent translocations falling within regions of significant 4C interactions. B) Venn diagram showing the number of unique and overlapping significant interchromosomal interactions for a window size of 200.

Task 2: Alter SATB1 expression to investigate the molecular basis of disrupted long-range interactions among breast cancer gene loci. (Months 12-24)

We have no data to report from this Task, which is scheduled to be undertaken this year.

Task 3: Use the high-resolution molecular assay Associated Chromatin Trap (ACT) to identify genes that physically interact with the selected breast cancer gene loci. (Months 12-24)

We have no data to report from this Task, which is scheduled to be undertaken this year.

KEY RESEARCH ACCOMPLISHMENTS

- Development of 4C-seq assays for breast cancer cells
- Demonstration that breast cancer cells differ from normal cells and from each other in their “interactome”

REPORTABLE OUTCOMES

- Manuscripts: none
- Licenses: none
- Degrees obtained: n/a
- Development of cell lines, tissue or serum repositories: none
- Informatics: new sets of data regarding interchromosomal interactions
- Funding applied for based on this award: none
- Employment or research opportunities: none

CONCLUSIONS

Physical contact is a prerequisite for chromosomal translocations. Both cytogenetic and molecular evidence suggests spatial proximity influences recurrent chromosomal translocations. From our data, we observed numerous breast cancer genes to be present within significantly interacting regions in normal breast cells. These data suggest the possibility that certain loci in the genome form “hubs” of preferentially interacting loci. These hubs may have a functional purpose, such as being co-transcribed in “transcription factories.” It is likely that these interacting genes regulate each others’ transcription and that changes in long range interactions in cancer may lead to detrimental changes in gene expression. Our studies will describe this breast cancer interactome, and it is possible that new gene targets for diagnosis or therapeutics may become evident from the study of interactome informatics.

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APPENDICES: none